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VASOCONSTRICTOR ACTIVITIES OF SOME NOVEL SYNTHETIC STEROIDS IN ALCOHOLIC SOLUTION

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Ethanolic solutions of 8 new, topically active, anti-inflammatory steroids and 1 standard (betamethasone 17-valerate) were assessed with a modified vascoconstrictor assay. Pallor was graded at 17 reading times for determination of complete blanching curves. The compounds were ranked by three methods: (1) summed % total possible score, (2) area under the blanching profile, and (3) square root transformation of sum of scores divided by number of volunteers, for statistical differentiation of the solutions.

Conclusions on structure–vasoconstrictor activity relationships were that substitution or removal of 21 hydroxy provided compounds with a wide range of activity. Poor activity correlated with a hemisuccinate salt grouping at position 21, or the absence of 11β-hydroxy.

The ability of topical steroids to produce skin blanching was first used as a method of comparison for these compounds by McKenzie and Stoughton [1], working with alcoholic solutions. Subsequent investigations [2–5] demonstrated a relationship between the most effective vasoconstrictors and clinical usefulness as topical anti-inflammatory agents. The original vasoconstrictor assay was refined by statistical analysis of results and by the use of a graded scoring system [6–8]. Barry and Woodford [9] assessed 30 proprietary preparations for bioavailability employing a graded estimation of blanching over several reading times to provide information in terms of area under the blanching curve and summed % total possible score and also used this technique to assess betamethasone 17-benzoate in gel and cream formulations [10]. This technique has been adapted here to assess 8 new steroid derivatives, synthesized on the basis of predicted structure–activity relationships, together with a standard, betamethasone 17-valerate.

MATERIALS AND METHODS

Steroids

The novel steroids (coded in Table I) were donated by Organon Laboratories Ltd. The betamethasone 17-valerate was a gift from Glaxo Laboratories Ltd. Steroids (structural formulae in Table I) were tested as received.

Solutions

Pure steroids were dissolved in absolute alcohol B. P. to provide solutions of 10^{-4} gm ml^{-1}, which were stored in coded, air-tight containers at 5°C. Thin-layer chromatography (TLC) and ultraviolet (UV) scanning enabled comparison of stabilities between freshly prepared solutions and samples aged for 8 months.

TLC. Two plates, 20 × 20 cm, were spread with a 0.25-mm layer of Kieselgel G type 60 (E. Merck) and activated at 110°–120°C 2 hr prior to use. Ten microliters of aged and fresh 10^{-4} gm ml^{-1} solutions of LH05 were applied alternately at 2-cm intervals to one plate, while WH14 solutions were applied to the other. The plates were placed in tanks saturated with toluene and ethyl acetate (1:1). The solvent front was allowed to rise to 2 cm from the top of the plates. After drying at room temperature for 5 min, the plates were sprayed with concentrated sulfuric acid (for LH05) or 2% v/v concentrated sulfuric acid in methanol (for WH14). They were then heated at 105° C for 5 min to develop the spots.

UV spectrophotometry. Diluted solutions were scanned directly against appropriate blanks, in a 1-cm cell, between 200 nm and 450 nm, in an automatic spectrophotometer (Pye-Unicam SP800).

Subjects

Ten volunteers were selected from a preliminary screening of 14 healthy persons, without reference to their sex or steroid sensitivity, but with reference to a consistent response to a standard preparation (betamethasone 17-valerate cream). None had received topical steroid application within the previous 3 months and none were tested during the trials at intervals of less than 1 month.

Methods

Vasoconstriction was assessed in a double-blind manner, solutions being applied from coded containers with reference to charts previously prepared from a random digits table; a total of 20 sites were used for each solution. This minimized natural variation of vasoconstriction at different sites. Ten microliters (± 0.16 μl) of the steroid solutions and of an alcohol blank were pipetted onto the flexor surface of each forearm into individual, 7-mm, silicone grease squares. These were stamped within circular punched, double adhesive-coated, Blenderm tape (3M Medical Products). Five milligrams of betamethasone 17-valerate cream was applied to a twelfth site, after rejection of the first gram of product obtained from the tube. After evaporation, the sites were occluded for 6 hr with type S12 μm Melinex polyester

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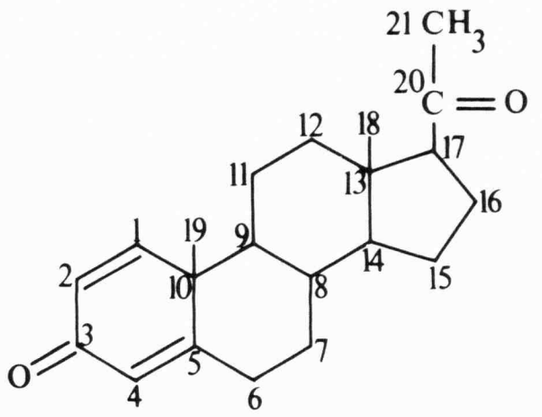
TABLE I. *Structural formulae of steroids*



Compound	Substituents				
	9[a]	11[b]	16[a]	17[a]	21
LHO2	—	O	CH_3	CH_3	$OCOCH_3$
LHO5[c]	—	OH	CH_3	CH_3	CH_3
LHO8	—	OH	CH_3	CH_3	$OCOCH_3$
Betamethasone 17-valerate	F	OH	βCH_3	$OCOC_4H_9$	OH
WH10	F	OH	CH_3	CH_3	OH
WH11	F	OH	CH_3	CH_3	$OCOC_2H_5$
WH12	F	OH	CH_3	CH_3	$OCOC_2H_4C_6H_5$
WH13	F	OH	CH_3	CH_3	$OCOC_2H_4COONa$
WH14	F	OH	CH_3	CH_3	C_2H_5

[a] All substituents in α position, except where stated.
[b] All substituents in β position.
[c] Δ 6.

film (ICI Plastics Division). Tapes and Melinex film were then removed, sites washed with soap and warm water (37°C) and dried gently.

In the first trial, blanching was assessed hourly between 6 to 13 hr and every 2 hours from 24 to 32 hr. In a second trial, readings were taken at 16, 18, 20 and 22 hr. Assessment was made in constant lighting conditions and without reference to the application charts, using a 0–4 scale with half-point ratings [9] as follows: 0 = normal skin; 1 = slight vasoconstriction; 2 = more intense vasoconstriction with at least 2 corners outlined; 3 = general, even vasoconstriction; 4 = more marked vasoconstriction with very distinct blanching. In each case, the first reading (6 hr) was made 10 min after removal of dressings so that any marginal erythema produced by this process had subsided and did not affect pallor assessment. During trials, volunteers were asked to avoid elevated temperatures and contact of water with their arms (e.g., humid atmospheres, strenuous exercise, hot baths, etc.).

RESULTS

Solutions examined by TLC gave single spots of similar intensity and with reproducible R_f values. There was no detectable peak shift nor decrease in peak height observed by UV scanning. It was therefore assumed that negligible decomposition had occurred in any of the solutions during storage.

So that results in this work could be related to any subsequent investigations, some method of standardizing response was sought. The results would then take into account variable factors such as time of year or change in panel composition. Barry and Woodford [9] evolved a method for unification of data, a modification of which was employed in this work. Betamethasone 17-valerate cream was applied as a standard to all volunteers. Summed % total possible score values were multiplied by the ratio: mean total values over selected reading times for betamethasone 17-valerate cream in previous trials/summed value over same reading times for betamethasone 17-valerate cream in this work.

As an example:

a. Mean of previous summed values (3 trials) for *betamethasone 17-valerate cream over* 6, 7, 8, 9, 12, 24, and 32 hr = 383.00%.

b. Summed value for betamethasone 17-valerate cream in this trial over 6, 7, 8, 9, 12, 24, and 32 hr = 398.75%.

c. Thus, present % total possible score values for all samples are multiplied by 383.00/398.75 = 0.9605.

Vasoconstrictor potency was compared using 3 parameters: (1) summed % total possible score, (2) area under the blanching profile, and (3) transformation of sum of scores divided by number of volunteers (Tm/10).

The summed % total possible score is defined as the sum, over all reading times, of volunteers' total score expressed as % of maximum possible score. As an example, the maximum score per site is 4, for 2 arms this would give 8, for 10 volunteers the total would be 80. In the 10-hr reading for WH11 the score of 55 is out of a possible 80, or 68.75%. This is standardized (68.75% × 0.9605) to 66.03%. This value is summed with the other values similarly obtained to give the summed % total possible score for WH11 of 432.23%. Values were similarly calculated for each steroid (Tab. II), which enabled ranking in order of vasoconstrictor efficacy.

Blanching profiles were constructed by plotting % total possible score as ordinate against time after application as abscissa (Figs. 1, 2). With exception of WH14, the nonfluorinated steroids (LH08, LH05, and LH02) peaked earlier than the fluorinated steroids by up to 4 hr. The absolute alcohol placebo was at all times under 10% total possible score and peaked earlier than the steroids, at 7 hr. Betamethasone 17-valerate cream and betamethasone 17-valerate solution produced strong vasoconstriction (peak values: 78.6% and 51.0%, respectively) with fairly well maintained response over the test period (32-hr values: 30.6% and 19.2%, respectively). Steroids LH08 andWH11 also showed quite marked peaks (61.6% and 66.0%, respectively) but pallor decreased more rapidly.

Area underneath the blanching profiles was obtained using a planimeter (Allbrit fixed index planimeter, short-arm Shandon model; W. F. Stanley); the areas (cm^2) were related to units equivalent to 1% total possible score for 1 hr, so that they could be expressed in "% total possible

TABLE II. *Blanching responses ranked with reference to area under the curve values*

Compound	Peak time (hr)	Area under the curve % × hr[b]	Blanching response[a]	
			Summed % total possible score[c]	Tm/10 mean values: square root transformation[d]
Betamethasone 17-valerate cream	11	1420	950	8.69
WH11	10	1087	734	7.63
WH10	11	938	607	6.88
Betamethasone 17-valerate in alcohol	12	901	582	6.78
LHO8	9	887	613	6.92
WH12	10	624	423	5.56
WH14	9	573	418	5.70
WH13	10–12	433	286	4.56
LHO5	8–9	346	256	4.47
LHO2	9	214	156	3.35
Absolute alcohol	7	82	61.8	1.77

[a] Values quoted to 3 significant figures.

[b] Obtained by planimetry of the blanching profile.

[c] The % total possible scores summed for all volunteers over all reading times.

[d] Square root transformation of sum of scores (Tm) divided by number of volunteers (10). The minimum significant range value k = 1.45 ($p < 0.05$), i.e., if the Tm/10 values of 2 preparations differ by more than 1.45, there is a significant difference between those preparations.

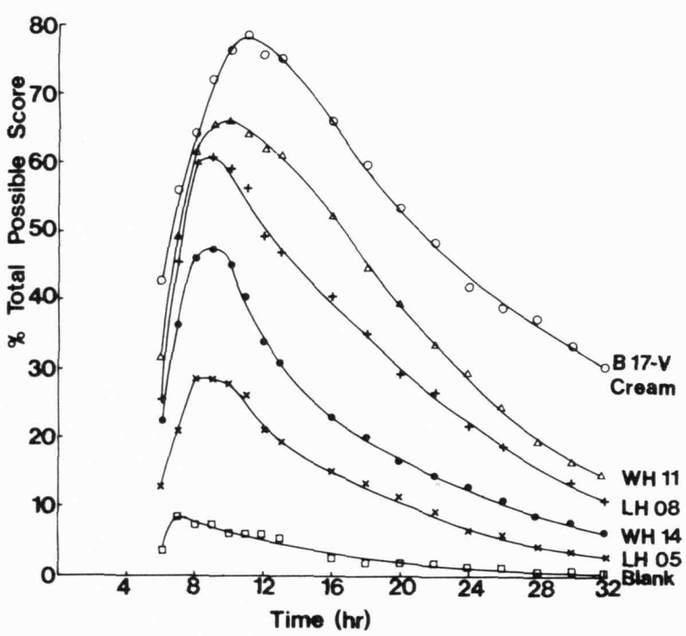


FIG. 1. Vasoconstriction profiles obtained after occluded application (6 hr) of betamethasone 17-valerate cream and ethanolic steroid solutions indicated.

score × hr" units. Table II shows the steroids arranged in decreasing order of area under the curve values, enabling comparison in biologic response-time units. It may be seen that the order is not exactly the same as for summed % total possible score ranking. LH08 had a score of 4% greater than WH10, but an area under the curve of 51% hr less. An identical decrease in area existed between WH12 and WH14 but the summed % total possible score dropped by only 1%.

The third classification of blanching results permitted statistical differentiation of the samples. The summed scores of each person for each sample were compared by two-way analysis of variance using an ICL4130 computer. The program provided for no transformation and 5 transformations of data—x^{-1}, $x^{-1/2}$, log x, $x^{1/2}$ and x^2 [11,12]—with subsequent tests for non-additivity [12,13] favoring the square root transformation. The analysis demonstrated statistically highly significant differences between preparations ($F = 42.68$, $p < 0.01$) and between the volunteers ($F = 6.69$, $p < 0.01$). Calculation of the minimum significant range (k) from the Studentized range test [14] enabled comparison of the steroids. Significant differences existed when the Tm/10 values (Table II) differed by more than k. As an example, at the 5% level, the nonsignificance values for WH11 were 7.63 ± 1.45, (6.19–9.08). Thus WH11 gave a significantly greater blanching response than steroids WH12 to LH02, inclusive, in Table II. Blanching due to absolute alcohol was significantly lower than that produced by any steroid.

DISCUSSION

In this work the standard McKenzie–Stoughton blanching test [1] has been refined by limiting occlusion to 6 hr and by increasing the frequency of observation. Similar modifications have been successfully employed by many other workers [5,7,9, 10,15]. Placebo sites were also included in the tests; these received alcohol only, which produced some blanching. The corresponding profile (Fig. 1) was of similar dimensions to that observed by Barry and Woodford [9] for a hydrocortisone formulation. This blanching may be due to increased hydration of the stratum corneum following occlusion rather than to any direct effect of the alcohol,

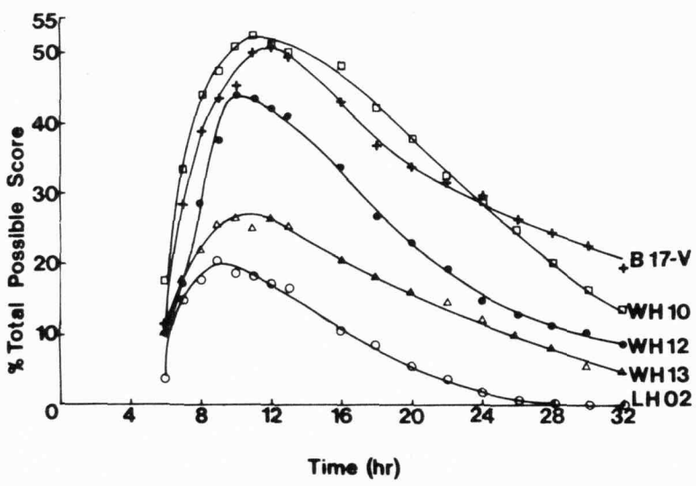


FIG. 2. Vasoconstriction profiles obtained after occluded application (6 hr) of ethanolic steroid solutions indicated.

although significant vasoconstriction to intradermally injected alcohol has been noted [15].

In the assessment of relative vasoconstrictor potencies, consideration was given to the fact that individual steroids have different blanching profiles and a single-point reading may be taken at a peak, or on either an ascending or descending portion of each graph.

Burdick, Haleblian, Poulsen, and Cobner [16] considered that area under the curve determinations of blanching profiles correspond to other pharmacokinetic methods used to estimate bioavailability from oral or parenteral preparations. These authors then, however, calculated summed % sites exhibiting vasoconstriction (from information obtained at only 3 reading times) for a number of compounds, relative to 0.05% fluocinonide ointment, and correlated the results with an area under the curve analysis. Similarly, summed % total possible score has been correlated approximately with area under the curve [17] and has been included in this work for comparative purposes (Table II). A criticism of this method is that there are often not enough readings to accurately characterize the curves; such a deficiency can cause reversal of a ranking order [9]. In this work, the number of readings were increased to 17 over a 26-hr period, but a ranking reversal still occurred between steroids WH10 and LH08.

In pharmacokinetics, "true" area under the curve correlates with the total amount of drug absorbed [18] and so the areas under the blanching profiles were assessed after conversion into correct response-time units (Table II), an approach based on the use of integral calculus to estimate the area of figures bounded in part by a regular curve. This accounts for both of the important parameters in a pharmacologic response—the intensity and the duration of action. This method also depends on readings of sufficient frequency to accurately draw each profile. It thus follows that the error in the area under the curve cannot be readily assessed unless numerous replicate full trials are performed. There is also a small error in adopting an upper limit of 32 hr instead of reading until all signs of blanching have disappeared in all volunteers.

Since Sulzberger and Witten [19] first used compound F (hydrocortisone) as a dermatologic agent, numerous modifications of the parent molecule have provided steroids with increased anti-inflammatory and low mineralocorticoid activities. All the steroids tested in the present study, except LH02, possessed the specific structural features which are now commonly considered desirable for topical activity, i.e., double bonds at C1 and C4, a C3 keto group, a β-hydroxy group at C11, β-methyl groups at C18 and C19, and a C20 keto group.

All the novel steroids possessed an α-methyl on C17 instead of the more usual α-hydroxy group. The subsequent decrease in water solubility should aid penetration by providing a more favorable partition coefficient and by decreasing chemical binding within the stratum corneum or hair follicles [20]. Responses of the C21 alkylated steroids, LH05 and WH14, were low. This may be attributed to the hydrophilic properties being too weak to maintain the drugs entirely in solution throughout percutaneous penetration. Masking of the C21 hydroxy group provided compounds of varying activity. WH10 has an unesterified C21 hydroxy group but was ranked below its propionate ester (WH11) *but above its phenylpropionate ester (WH12). It was a considerably more effective vasoconstrictor than WH14, in which the C21* hydroxy had been replaced by an ethyl group. The weaker activities of LH05, LH02, and WH13 correlate respectively with absence of C9 α-fluoro and presence of a double bond at C6 (which would probably increase molecular dipole moment in the plane of the rings and thus decrease mobility), absence of the C9 α-fluoro and the C11 β-hydroxy group, which has been implied as a primary attachment point of steroid to receptor [21] and strong ionic character of the C21 hemisuccinate salt, which would increase binding in the stratum corneum and reduce drug mobility.

Approximately one-third of currently available topical steroid formulations are fluorinated, usually in the C6 and/or C9 α positions. Rein, Fleischmajer, and Rosenthal [22] clinically evaluated a new topical, fluorinated steroid—triamcinolone diacetate. Since then, other fluorinated compounds such as fluocinolone acetonide, betamethasone 17-valerate, and fluclorolone acetonide have proved popular, topical therapeutic agents. However, recent trends are toward the use of effective, nonfluorinated steroids, where possible [23–26]. In the present work, the nonfluorinated steroid LH08 gave a blanching response superior to 3 of the novel, fluorinated steroids by area under the curve assessment.

Blanching results, however, should be interpreted with caution, as it has been found in comparative blanching tests with formulated ointments and creams that steroids in 100% alcohol did not provide maximal responses (unpublished data). This was probably due to precipitation of the steroid [27]. A thin layer of crystals on the skin would need redissolution, with a subsequent decrease in steroid penetration and vasoconstriction. Addition of a small percentage of nonvolatile solvent may well increase blanching scores.

Selection of steroids for clinical use will involve considerations not dealt with in this study. The toxicology of the drugs requires investigation (e.g., effect of overdosage, excessive effect at normal dosage, unwanted side effects, hypersensitivity reactions and idiosyncrasies, if any). An awareness of drug interactions, outside or within the body, which may have diverse effects may influence selection. Even though there is no irrefutable evidence that vasoconstriction and anti-inflammatory efficacy are directly related (merely a series of very strong analogies), the vasoconstrictor assay is an extremely valuable method for rapid, preclinical assessment of potential topically active ste-

roids. After giving due consideration to the physico-chemical characteristics of the steroids and their onset of action, duration of action, and maximum intensities from alcoholic solution, the correct vehicle for each drug should be selected. Each formulation must be stable upon storage and the drug must be compatible with individual ingredients of the base, so that a low, working concentration of the drug can provide optimum bioavailability. This implies that the steroid should possess maximum thermodynamic activity in the topical vehicle so that it is readily released from the base into the skin. All these factors will affect the final choice of a steroid to be submitted to clinical trial.

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